Sex-specific expression of a novel gene *Tmem184a* during mouse testis differentiation

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Abstract

During mouse embryogenesis, the fate of the bipotential gonads is sealed around 10.5 days *post coitum* (dpc) when the Y-linked gene *Sry* specifies the differentiation of testes in males, whereas in females, absence of *Sry* results in ovary formation. Apart from the pivotal action of *Sry*, many other genes are known to be involved in sex determination and subsequent differentiation. Much is still unknown regarding the regulatory hierarchy governing these events and many more sex differentiation genes are yet to be discovered. In this study, we investigated the expression of *Tmem184a*, a novel gene encoding a protein of unknown function, but with predicted kinase activity, during mouse embryogenesis. We show that *Tmem184a* is expressed at high levels in the developing testis from 11.5 dpc, a time of active proliferation and differentiation. *Tmem184a* expression is further shown to be expressed exclusively within the Sertoli cells of the developing testis cords, suggesting that it may mediate sex-specific signaling events during Sertoli cell differentiation.

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Introduction

In most mammals, sex is determined by the function of *Sry*, a gene that resides on the Y chromosome. In mice, *Sry* expression is induced within XY genital ridges around 10.5 days *post coitum* (dpc; Koopman *et al.* 1990), after which the XY gonad enters a stage of active differentiation (reviewed by Brennan & Capel 2004). The developing testis is most notably distinguishable from the more latent ovary during this period by the formation of testis cords. This process involves dramatic tissue reorganization and proliferation, along with recruitment of several cell lineages that migrate into the gonad from the adjacent mesonephros and the coelomic epithelium (Martineau *et al.* 1997, Karl & Capel 1998, Val *et al.* 2006). By 12.5 dpc, roughly 48 h after the initiation of *Sry* expression, the testis becomes compartmentalized into cords, which consist of germ cells and supporting Sertoli cells encapsulated by a thin layer of peritubular myoid cells, and the interstitium composed of endothelial, Leydig, and connective tissue cells.

Of the various cell lineages making up the testis, only the Sertoli cells express *Sry* (Albrecht & Eicher 2001, Sekido *et al.* 2004). Sertoli cells are known to influence

differentiation of other testicular cell types and thus play a critical inductive role in testis development (Burgoyne *et al.* 1988; reviewed by Wilhelm & Koopman 2006). One of the first critical steps in the testis-determining pathway is activation of *Sox9* (Vidal *et al.* 2001, Chaboissier *et al.* 2004, Barrionuevo *et al.* 2006). However, the genetic pathways downstream of *Sry* that regulate sex determination and Sertoli cell differentiation remain largely unclear.

To further understand the regulatory pathways governing these processes, it is necessary to identify novel genes involved in sex-specific differentiation of the gonad. Many researchers have used expression-screening strategies, including microarray studies, in an effort to identify a plethora of genes showing sexually dimorphic expression in gonads. We decided to further explore the spatiotemporal expression pattern of a novel gene with accession no. BC019731, which was shown in two such studies to be up-regulated at 11.5 dpc XY gonads when compared with XX gonads (Nef *et al.* 2005, Beverdam & Koopman 2006).

Tmem184a spans a 9.3 kb region on chromosome 5 and was initially identified in a mouse cDNA library

screen (Strausberg *et al.* 2002), with subsequent updated annotations (Nef *et al.* 2005). TMEM184a has a predicted kinase activity; however, its amino acid sequence does not align to any known domains and hence the function of this protein is unknown. Mouse *Tmem184a* has putative orthologs in a number of mammalian species, including human (*Homo sapiens*; Q6ZMB5), chimpanzee (*Pan troglodytes*; LOC735493), dog (*Canis familiaris*; LOC489896), and rat (*Rattus norvegicus*; RGD1306702). Homology between these putative orthologs is more than 80%, and hence sufficiently high to be confident that the genes are orthologs and potentially have conserved function across species. In mouse, only one paralogous gene (4732495E13Rik/*Tmem184b*) has been identified (sharing putative domains of around 80% homology), with the two gene products making up a predicted MAP kinase-activating protein family of two members.

The involvement of kinase regulatory pathways during testis differentiation is poorly understood and has the potential to shed light on several unexplained aspects of these morphological events. As a first step toward elucidating the role of *Tmem184a* during mouse developments, we studied in detail its expression pattern and found it to be specifically up-regulated within the testis shortly after gonadal sex determination, with continued testis-specific expression into adulthood. The restricted expression of this gene in Sertoli cells suggests a role for the encoded protein in the development and/or continued function of that cell type.

Materials and Methods

Animals

Mouse embryos were collected from timed matings of the CD1 outbred strain and the W^e mutant inbred strain, with noon of the day on which the mating plug was observed designated 0.5 dpc. Embryos at 11.5 dpc or younger were sexed by RT-PCR using *Zfy* gene-specific primers *Zfy* F: 5'-CCTATTGCATGGACTGCAGCTTATG and *Zfy* R: 5'-GACTAGACATGTCTTAACATCTGTCC. Older embryos were sexed by morphological assessment of gonads.

RNA isolation, cDNA synthesis, and SYBR green real-time RT-PCR

Fetal gonads were collected by dissection, pooled according to sex and developmental stage (one litter per pool), and total RNA was isolated using the SV Total RNA Isolation System (Promega) as per manufacturer's instructions. cDNA was synthesized from 1 µg total RNA by RT (Superscript III, Invitrogen) using random primers (Promega) according to manufacturer's instructions.

Relative cDNA levels were analyzed by quantitative real-time RT-PCR (qRT-PCR) using an ABIPrism-7000 Sequence Detector System.

For statistical significance, all qRT-PCR experiments were performed in triplicate and repeated thrice on separate biological samples, each representing one pooled litter (7–10 littermates/sex), and finally represented as mean $\pm$ S.E.M. of the three individual experiments. Samples were analyzed in 25 µl reactions containing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 3.75 µM each forward and reverse primers, and 1 µl cDNA, prepared as described using an ABIPrism-7000 Sequence Detector System. PCR was conducted over 40 cycles of 95 °C for 15 s and 60 °C for 1 min in a two-step thermal cycle, preceded by an initial 10-min step at 95 °C to activate the Amplitaq Gold DNA polymerase. The primers were designed using the Universal Probe library tool (<http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp>) as previously described (Beverdam & Koopman 2006) and for this study, the primers used were rtTmem184a.F: 5'-CTGCATTGTGAAACCCGTTA and rtTmem184a.R: 5'-GGTAGCCGCTGTGGATGT; rtSox9.F: 5'-AGTACCCGCATCTGCACAAC and rtSox9.R: 5'-TACT-TGTAATCGGGGTGGTCT; and rtOct4.F: 5'-TGC-GGAGGGATGGCATACTG and rtOct4.R: 5'-GCACA-GGGCTCAGAGGAGGTTC. To adjust for variation in input cDNA, samples were normalized against 18S RNA using the Δ Ct method. 18S primers used were rt18S.F: 5'-GATCCATTGGAGGGCAAGTCT and rt18S.R: 5'-CCAA-GATCCAACCTACGAGCTTTT. Dissociation curve analyses were performed.

Whole-mount and section in situ hybridization (WISH/SISH)

Embryos and dissected gonads/mesonephroi were fixed in 4% paraformaldehyde (PFA) in PBS for several hours at 4 °C. WISH with digoxigenin (DIG)-labeled RNA probes was carried out essentially as described by Hargrave *et al.* (2006). For SISH samples, whole embryos were processed and mounted in paraffin wax and stored at 4 °C. SISH was performed on 7 µm sagittal sections that were dewaxed, rehydrated, and incubated in 5 µM/ml proteinase K for 20 min at room temperature (RT), after which samples were washed in PBS. Sections were re-fixed with 4% PFA for 10 min at RT, acetylated, and pre-hybridized with hybridization solution (50% formamide, 5× SSC, 5× Denhardt's solution, 250 µg/ml yeast RNA, and 500 µg/ml herring sperm DNA) for 2 h at RT. Hybridization (0.5 µg/ml probe in hybridization solution) was performed overnight at 60 °C. Slides were washed in 5× SSC for 5 min, 0.2× SSC for 1 h at 60 °C, 0.2× SSC for 5 min at RT, and NT buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5)) for 5 min at RT, before blocking for 2 h with blocking solution (10% heat-inactivated sheep serum in NT buffer) in a humidified chamber. Anti-DIG antibody (Roche) in

blocking solution (1:2000) was added to the slides and incubated overnight at 4 °C. Unbound antibodies were removed by washing thrice in NT buffer. Section were equilibrated in NTM buffer (100 mM NaCl, 100 mM Tris-HCl (pH 9.5), 50 mM MgCl₂) and incubated in color solution (3.5 µl BCIP (Roche), 3.5 µl NBT (Roche) per ml NTM buffer) until staining was detected.

A 273 bp *Tmem184a* fragment was cloned from position 419–692 (accession no. BC019731) into pGEM-T Easy vector (Promega). Primers used were *Tmem184a*.F: 5'-TTCCCCAGCCAGGTGTATTTCAG and *Tmem184a*.R: 5'-TGCGTTTCTCTATGTTGCGACTC. The probes for *Amh*, *Sox9*, and *Oct4* were made as previously described (Münsterberg & Lovell-Badge 1991, Wright *et al.* 1995, Schepers *et al.* 2003).

Results

Embryonic *Tmem184a* expression

To study the expression pattern of *Tmem184a* during embryogenesis, we first conducted qRT-PCR and SISH analyses on 13.5 dpc mouse embryos and tissues. qRT-PCR assays revealed higher levels of *Tmem184a* transcripts in the testis when compared with ovary, brain, heart, liver, lung, limb, and kidney (Fig. 1A). Low levels of expression were detected within the ovaries, liver, and lung, but no expression was detected in the remaining tissues. SISH on whole embryos confirmed the qRT-PCR data, with a clear and specific hybridization signal restricted to the testis (Fig. 1B).

Figure 1 Testis-specific expression of *Tmem184a* in the mouse embryo. (A) qRT-PCR analysis of *Tmem184a* mRNA expression relative to 18S RNA (mean $\pm$ S.E.M. of three independent experiments, each performed in triplicate) in 13.5 dpc mouse embryonic tissues. For each experiment, non-gonadal tissues were pooled from at least three littermate embryos, and testes and ovaries with attached mesonephroi were pooled from at least six littermates. *Tmem184a* shows high expression in testis and only weak expression in liver, lung, and ovary. No expression was detected in other tissues examined. (B) DIG-labeled sagittal SISH on 13.5 dpc male embryo. Strong *Tmem184a* expression is evident in the testis when compared with other tissues and (C) expression is restricted to the testis cords. (D) No staining was evident with a *Tmem184a* sense riboprobe. G, gonad; M, mesonephros. Scale bar = 1 mm (B); 200 µm (C and D).

Temporal profile of *Tmem184a* expression in the developing gonads

Next, we studied the temporal expression profile of *Tmem184a* at 10.5–16.5 dpc in the gonads of both sexes (Fig. 2A). *Tmem184a* mRNA was up-regulated in the testes from 11.5 dpc, with roughly fourfold higher levels of transcription detected relative to the levels of transcription in ovaries at this stage. Expression levels continue to increase in the testes until 16.5 dpc, whereas they remain low in the ovaries during the same period of time. WISH analyses confirmed this temporal expression pattern (Fig. 2B), with a weak signal detected in the testes at 11.5 dpc, becoming increasingly intense at 12.5 and 13.5 dpc. In the ovaries, only a weak staining was observed at these latter stages (Fig. 2B).

Cell-specific *Tmem184a* expression within the fetal testis

To identify the cell types that express *Tmem184a*, we performed SISH using specific probes for *Tmem184a*, the Sertoli cell marker *Amh*, and the germ cell marker *Oct4* (Fig. 3). Sertoli cell nuclei are located peripherally in the developing testis cords, but their cytoplasm projects toward the center of the cords, surrounding the germ cells. Their cytoplasm is therefore stellate, in contrast to the characteristic annular cytoplasm of the germ cells that are concentrated toward the lumen of the testis cords. A stellate hybridization pattern was observed with both the *Tmem184a*- and *Amh*-specific probes typical of Sertoli cell gene expression, and clearly differs from the pattern of *Oct4* probe hybridization (compare Fig. 3 A, D/

Figure 2 Temporal expression analysis of *Tmem184a* in the fetal gonads. (A) qRT-PCR analysis of *Tmem184a* mRNA expression relative to *18S* RNA (mean $\pm$ S.E.M. of three independent experiments, each performed in triplicate) showing male-specific up-regulation in fetal gonads from 11.5 dpc. Individual experiments were performed on RNA obtained from pooled gonads from at least six littermates. Stage 10.5 and 11.5 dpc gonads had mesonephroi attached, whereas later-stage gonadal samples had mesonephroi removed. (B) WISH analysis confirming male-specific up-regulation of *Tmem184a* in fetal gonads from 11.5 dpc. A weak signal was evident in ovaries from 12.5 dpc, but with a markedly stronger signal in the testis. Dotted lines mark the mesonephric–gonadal boundaries. M, mesonephros; G, gonad.

Figure 3 Cell-specific *Tmem184a* expression in the fetal male gonad. SISH staining of 13.5 dpc male gonads using specific probes for *Tmem184a* (A and D), the Sertoli cell marker *Amh* (B and E), and the germ cell marker *Oct4* (C and F). *Tmem184a* is expressed in the testis cords and localizes to Sertoli cells. *Tmem184a* is not expressed in germ cells with staining different to that observed for the germ cell marker *Oct4*, as evident by the opposing staining between *Tmem184a/Amh* and *Oct4* within the same testis cord (compare D/E with F). Boxed areas in A, B, and C indicate regions shown in D, E, and F respectively. Scale bar = 100 μ m (A–C); 25 μ m (D–F).

B, E and C, F). *Tmem184a* was not detected in any other cell types of the testis, including peritubular myoid cells, and therefore appear to be specific to Sertoli cells.

To further characterize the cell-specific expression of *Tmem184a* within the developing testis, qRT-PCR analyses were first performed on RNA collected from wild-type and W^e/W^e XY gonads, which are devoid of germ cells (Buehr *et al.* 1993; Fig. 4A). Similar to the expression of the Sertoli cell-specific gene *Sox9*, *Tmem184a* expression persists in the W^e/W^e gonads when compared with wild-type littermates. In contrast, expression of the germ cell marker *Oct4* was lost in the W^e/W^e gonads as expected. The apparent increase in the expression levels of both *Sox9* and *Tmem184a* in W^e/W^e XY gonads when compared with wild-type littermates is likely due to the increased overall proportion of somatic cells in the absence of germ cells in W^e/W^e XY gonads. WISH experiments confirmed the persistent expression of *Tmem184a* in the testis cords of W^e/W^e XY gonads (Fig. 4B). These data clearly show that *Tmem184a*

expression is not dependent on the presence of germ cells and is associated with somatic cells.

Adult *Tmem184a* expression

Finally, we performed qRT-PCR analyses on adult mouse tissues to establish whether *Tmem184a* remained up-regulated in the adult testis. As in fetal tissues, the expression levels of *Tmem184a* in adult testis were far higher than those in other tissues examined (Fig. 5A). SISH experiments further confirmed persistent *Tmem184a* expression in adult testis with *Tmem184a* expression pattern similar to that of the Sertoli cell marker *Sox9* (Fig. 5B–E), with both gene transcripts detected in the periphery of the testis cords. Close examination of sections indicated a lack of *Tmem184a* expression in peritubular myoid cells, and the expression was Sertoli cell-specific.

Discussion

In the present study, we have analyzed the expression pattern of a novel gene, *Tmem184a*, during mouse

Figure 4 Germ cell-independent *Tmem184a* expression in 13.5 dpc testes. (A) qRT-PCR analysis of total RNA extracted from W^e/W^e versus pooled wild-type (+/+) and heterozygous (+/ W^e) littermate gonads using gene-specific primers for *Tmem184a*, *Sox9* (Sertoli cell marker), and *Oct4* (germ cell marker) relative to 18S RNA (mean $\pm$ s.e.m. of three independent experiments). Individual experiments were performed in triplicate on RNA obtained from pooled gonads from 3 to 4 littermates. *Tmem184a* shows similar expression to *Sox9* and is maintained in germ cell-depleted gonads. In comparison, *Oct4* expression was lost in the W^e/W^e samples. (B) WISH analysis comparing *Tmem184a* gene expression in W^e/W^e and wild-type (+/+) male gonads. *Tmem184a*-specific staining is seen in both wild-type and littermate W^e/W^e gonads (wt, wild-type (+/+) and heterozygous (+/ W^e) littermates; we, homozygous W^e/W^e mutants).

Figure 5 Testis-specific expression of *Tmem184a* in the adult mouse. (A) qRT-PCR analysis of *Tmem184a* mRNA expression relative to *18S* RNA (mean $\pm$ S.E.M. of three independent experiments, each performed in triplicate) in adult mouse tissues. The three independent biological samples represent two female and one male adult CD1 mouse, with the gonadal tissues represented by three individual ovaries and testes. *Tmem184a* shows high expression in testis and only weak expression in ovary, liver, lung, limb, and kidney. No expression was detected in the adult heart. One brain sample showed low levels of expression, but no expression was detected in the other two samples examined. ISH staining using specific probes for *Sox9* (B and C) and *Tmem184a* (D and E) shows a continued Sertoli cell-specific expression in the adult testis. Boxed areas in B and D indicate regions shown in C and E respectively. Scale bar = 50 μ m (B and D); 25 μ m (C and E).

development and demonstrate a specific up-regulation in the Sertoli cells of the testis. *Tmem184a* expression is activated in XY gonads from 11.5 dpc onwards, ~24 h after the onset of *Sry* expression and coincident with the up-regulation of *Sox9* (Kent *et al.* 1996, Morais da Silva *et al.* 1996). This expression profile observed in early gonadal development strongly suggests a role for *Tmem184a* in testis differentiation.

The 449 amino acid TMEM184A protein contains seven putative transmembrane helices, strongly suggesting a role in transmembrane signaling pathways acting in or on developing Sertoli cells. It is possible that *Tmem184a* may mediate signaling between germ cells and Sertoli cells, or between other somatic cells and Sertoli cells, or alternatively within regulatory pathways governing architectural compartmentalization of the testis. In addition, the expression of *Tmem184a* in the adult testis suggests a continuing role for TMEM184A function. Although detectable in other tissues in both fetal and adult mice, the expression of *Tmem184a* was significantly higher in testes, strengthening the concept that TMEM184A may elicit specific functions limited to the testis, potentially mediating a male-specific cascade of phosphorylation during Sertoli cell differentiation and/or function. In addition, the restricted expression pattern of *Tmem184a* makes this gene an excellent target for functional analysis by gene targeting in mice.

Due to the observed tissue-specific expression of *Tmem184a* and its up-regulation from 11.5 dpc, we hypothesize a close transcriptional regulation with the testis-determining factor *SRY*, either directly or indirectly. After the onset of *Sry* expression in the pre-Sertoli cell lineage around 10.5 dpc, the testis enters an active phase of proliferation and differentiation, which dictates both inter- and intracellular signaling pathways, almost certainly involving kinase pathways and phosphorylation cascades. However, as *Tmem184a* expression persists into adulthood, we hypothesize that TMEM184A function is distinct from sex determination, but is integral to testis differentiation. Further studies will focus on determining protein structure and sub-cellular localization in order to better understand the likely cellular functions of TMEM184A protein. It will be especially beneficial to elucidate whether TMEM184A is a cellular transduction molecule or kinase receptor, ultimately allowing for extensive *in vitro* and *ex vivo* studies after the characterization of effector molecules.

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